Electron Capture Dissociation of Gaseous Multiply Charged Ions by Fourier-Transform Ion Cyclotron Resonance

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Fourier-transform ion cyclotron resonance instrumentation is uniquely applicable to an unusual new ion chemistry, electron capture dissociation (ECD). This causes nonergodic dissociation of far larger molecules (42 kDa) than previously observed (<1 kDa), with the resulting unimolecular ion chemistry also unique because it involves radical site reactions for similarly larger ions. ECD is highly complementary to the well known energetic methods for multiply charged ion dissociation, providing much more extensive protein sequence information, including the direct identification of N- versus C-terminal fragment ions. Because ECD only excites the molecule near the cleavage site, accompanying rearrangements are minimized. Counterintuitively, cleavage of backbone covalent bonds of protein ions is favored over that of noncovalent bonds; larger (>10 kDa) ions give far more extensive ECD if they are first thermally activated. This high specificity for covalent bond cleavage also makes ECD promising for studying the secondary and tertiary structure of gaseous protein ions caused by noncovalent bonding. (J Am Soc Mass Spectrom 2001, 12, 245–249) © 2001 American Society for Mass Spectrometry

Fourier-transform ion cyclotron resonance (FTICR) mass spectrometry [1] has revolutionized many research areas of gaseous ion chemistry, as described in other reviews of this special issue. Since 1984 our Cornell laboratory has used the unique FTICR capabilities of high resolution, simultaneous data collection, and tandem MS (MS/MS, MS^n) for biomolecule characterization [2]. Until recently, our FTICR research in ion chemistry to provide primary structure information has focused on the unimolecular dissociations of ionized proteins and DNA using a variety of methods for vibrational excitation. These include collisional activation [3], laser infrared multiphoton dissociation [4], blackbody infrared dissociation [5], and surface induced dissociation [6]. However, all of these result in essentially the same unimolecular chemistry for large biomolecules, as their unusually large number of vibrational degrees of freedom must all be activated in the "ergodic" energizing process, primarily dissociating those bonds of lowest activation energy. Even photodissociation of multiply charged protein ions with 193 nm photons (6.4 eV) from an excimer laser produces the same cleavages [7]; the large amount of energy deposited by a single photon is also randomized over the many vibrational degrees of freedom before dissociation takes place. Substituents added in post-translational modifications, such as glycosylation, carboxylation, oxidation, and phosphorylation, often dissociate with even lower activation energies than those of backbone cleavages [8]. Here we describe the new ion chemistry resulting from electron capture dissociation (ECD) of multiply charged cations [9] for which FTMS provides ideal instrumentation. ECD is a nonergodic technique [10] that thus not only provides information on the primary protein structure that is highly complementary, but also information on secondary and tertiary structure because ECD of noncovalent bonds is unfavorable [9d].

Proteomics is suddenly a highly important research area, resulting from the revolutionary elucidation of DNA sequences of whole genomes. These, estimated to be 80,000 for humans, encode the primary sequences of the organism's proteins. For this problem, mass spectrometry has shown great promise, with a "bottom up" approach using mass spectrometry and MS/MS of low (<2 kDa) MW proteolysis products [11] and a "top down" approach of MS^n of the protein and its larger fragments [12].

Mass spectrometry identification of a specific residue (amino acid) in a linear protein chain requires at least

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two mass values, one each from dissociation of the bonds on either side of the residue, preferably a mass representing a fragment that still contains one of the termini [11, 12]. Because the conventional energizing methods favor dissociation of the lowest energy bonds [3–7], these methods seldom provide complete MS/MS sequence information for proteins larger than 1 or 2 kDa. Chemical [11] or ion–molecule [13] modifications can change the reactivity adjacent to specific amino acids of a protein, but a general sequencing method would require a multiplicity of such methods. Some years ago it was suggested that neutralization of a protonated site would form a hypervalent species that should have an unusually low dissociation energy for backbone protein cleavage [14]. However, all efforts to exploit this new chemistry failed, only to find that related chemistry was basic to the serendipitous discovery of electron capture dissociation (ECD) [9]. Experimental methodology for ECD will not be reviewed here, as this has been covered in detail recently [9c, d].

Selective Radical Ion Chemistry of Large Multiply Charged Ions

The energetic methods [3–7] for backbone dissociations of multiply charged proteins almost all involve cleavage of the amide bond (eq 1) to form ***b***, ***y*** ions. However, after electron capture at a

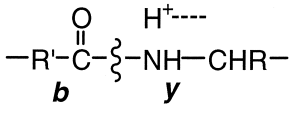
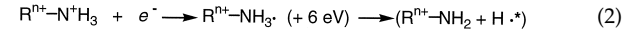
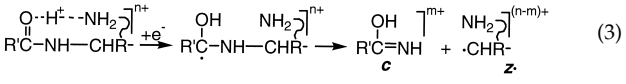


Diagram 1

protonated site to form a hypervalent species (eq 2), the major ECD pathway involves N-alkyl bond cleavage (eq 3) to form ***c***, ***z*·** product ions, with a minor pathway to form ***a*·** , ***y*** ions (eq 4).



(2)



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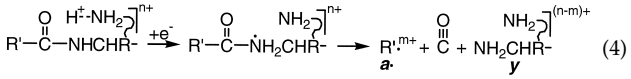
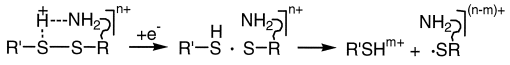


(4)

Diagram 2

ECD ion chemistry is unique for large molecules in that H· capture produces an odd-electron ion ($OE^{n+\cdot}$) leading to a *radical site reaction*; almost all "soft ionization" techniques produce even-electron (EE^{n+}) large ions. For the small $OE^{n+\cdot}$ ions formed by higher energy (~70 eV) electron ionization (EI), such radical site reactions have been extensively studied [15]. Their far greater utility for structural characterization has made EI MS the dominant method for identifying "global" unknowns over many decades [16]. Thus the main ECD reaction of protein ions (eq 3) parallels the common "α-cleavage" reaction in that it "donates an electron to form a new bond to an adjacent atom concomitant with cleavage of another bond to that atom, moving the radical site" [15]. Also the bond dissociation energy for this $OE^{n+\cdot}$ ion unimolecular decomposition is far lower than that for the amine NH–C_α cleavage of an EE^{n+} ion [17–19].

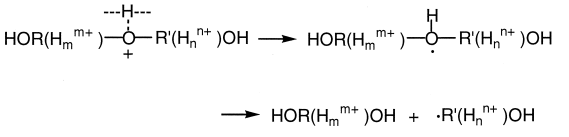
H· capture can also form another hypervalent species by attack on a saturated heteroatom, followed by cleavage of another bond to the heteroatom (eq 4). This is especially favored for disulfide bonds (eq 5), as these have an unusually high hydrogen



(5)

Diagram 3

atom affinity [9b]. ECD is especially useful for locating posttranslational modifications of proteins because the H· affinity of the amide carbonyl is substantially higher than that of common side chain modifications, such as glycosylation, carboxylation, oxidation, and phosphorylation [8]. However, if such hydroxy and ether oxygens are the only hetero atoms present, as in polyethylene glycol ions, they can capture H· and lead to ECD of the hypervalent new species (eq 6) [20]. Polyester multiply charged ions also capture



(6)

Diagram 4

electrons, but at present this has led to minimal fragmentation. An important, and obvious, limitation to this ion chemistry is that ECD is not applicable to negative ions or to singly charged positive ions.

Additional Selectivity through Nonergodic Dissociation

Removing the unpaired electron from this multiply charged ion should require 5–7 eV of energy; conversely, the electron addition that formed this hypervalent species should also add 5–7 eV of energy. Randomization of this energy over the thousands of degrees of freedom of a large protein ion would result in only

millivolts of excitation per bond. However, the unusual proportion of backbone bonds cleaved (eqs 3 and 4), plus extensive other evidence [9], indicates that these cleavages are nonergodic, caused by this large addition of energy before it is randomized away from the reaction site. The recent scientific breakthroughs of femtosecond spectroscopy have greatly popularized these reactions that take place in such a time domain, but nonergodic dissociations have previously been studied only for small molecules, with the acetone$^{+\cdot}$ as the largest ion exhibiting this dissociation [10].

The electron captured may initially form a high Rydberg state, which then undergoes surface crossing involving an initial lowest energy geometry of the protonated amine and amide carbonyl to yield the final state reactants of eqs 3 and 4 [9b, c]. This surface crossing to form ***c***, ***z***$\cdot$ ions converts the carbonyl double bond to a nominal single bond, so that there should be a substantial Franck–Condon factor that also favors nonergodic cleavage. Despite the infrequent H^+ solvation to a disulfide (eq 6) in a dynamic protein ion, this crossing should be far more favorable, as S–S has a far higher H$\cdot$ atom affinity. ECD of a 10 kDa protein with one –S–S– bond gave only products from cleavage of that bond [9b].

Thus capture of one electron cleaves only one covalent bond. Capture of a second electron by an ECD product is only observed with nearly complete reduction of the precursor ion [9d], as the e^- capture cross section is proportional to the square of the charge. This makes negligible any rearranged products due to ECD cleavage of the two bonds, such as $[CH_3(C_2H_4O)_xCH_3 + nH]^{n+} \rightarrow [CH_3(C_2H_4O)_yCH_3 + nH]^{n+}$ with loss of internal monomer units, while >20% of CAD products show this rearrangement. This is uniquely useful for MS/MS characterization of separated copolymer oligomer formulas of only 0.5% relative abundance [21].

Because ECD occurs without an appreciable increase in the average internal energy of the molecule, there is little cleavage at sites of low H$\cdot$ affinity, even at hydrogen bonds and other weak noncovalent bonds that are the basis for the secondary and tertiary conformational structure in the ions [22]. Ergodic backbone dissociation methods [3–7] that add energy randomly over the ion dissociate the noncovalent bonds first [22, 23], eliminating the secondary/tertiary structure. However, this extra cyclic structure is a problem for sequencing by ECD, as backbone cleavage will not produce two lower mass products if they are still held together by such noncovalent bonds. Normal ECD has not produced fragment ions in protein ions that are larger than ~20 kDa [9a–c]. However, combining ECD with conventional collisional [3, 6] or infrared [4, 5] activation gives extensive "Activated Ion" (AI) ECD spectra for protein ions as large as 42 kDa, and increases the number of cleavages of apo-myoglobin ions (17 kDa) from 33 to 99 [9d].

Conformational Characterization

As a silver lining to this cloudy nature of ECD, the difference between a conventional and AI ECD spectrum of the same ions must mainly arise from the ions' conformational structure. Research in progress [24] is finding that these ECD differences can provide unique secondary/tertiary structural details for a number of protein ions. For example, ECD spectra are effected dramatically by BIRD [5] at different temperatures or IRMPD [4]. Fast (<1 s) unfolding by laser IR irradiation makes possible the measurement of refolding kinetics of half lives of seconds to minutes [25]. In addition, ECD characterization of noncovalent intermolecular adducts formed by ESI should provide important information on the similarity of their solution and gas phase structures, on which a variety of opinions have been expressed [22, 26].

Extensive Backbone Bond Cleavage of Proteins

A good correlation is observed between the site of ECD and hydrogen atom affinity [9]. In the absence of higher affinity species, such as S–S bonds (eq 5) or Fe^{3+}, or local stabilization by noncovalent bonding (vide supra) [9d], there is surprisingly little difference in the ECD cleavage tendencies of the common amino acids [17], consistent with comparable H$\cdot$ affinities for most amide carbonyl groups (eq 3). Exceptions are the C-terminal side of Trp, with a higher tendency because of its 3-alkylindole side chain [9b], and the N-terminal side of Pro, which does not separate into products after undergoing eq 3 because of the remaining bond due to its cyclic structure [9] (it can yield ***a***$\cdot$, ***y*** products by eq 4, however).

This great advantage of ECD in producing a very large number of products brings with it the instrumental challenge of measuring a far greater number of mass values. Producing *c* and *z*$\cdot$ product ions from cleavages between all residues of a 101 amino acid protein (~12 kDa) would yield 200 different isotopic clusters, plus those from ***a***$\cdot$, ***y*** products and products formed in other charge states. Fortunately, FTICR has unique capabilities for simultaneous ion measurements. Our 6 tesla instrument with the THRASH data reduction program [18] has, for example, provided mass values for nearly 400 isotopic clusters from an ECD spectrum of the 104-residue cytochrome *c*; more than 800 were provided from an ESI spectrum measured on the 9.4 tesla Marshall instrument [12d]. Thus a basic limitation to the extent of sequence information in larger proteins is the signal/noise (S/N) achieved for the determinant fragment ions. Multiple spectra are an aid to this problem; relative ECD product abundances are a function of charge state [9], secondary/tertiary structure (gaseous noncovalent bonding), and electron current [9d], so that several ECD (and CAD) spectra measured under different conditions provide more extensive sequence infor-

mation, as well as improved S/N. Product ions representing cleavages between all 76 amino acids of ubiquitin (8.6 kDa) were found in two ECD and one CAD spectrum; submitting all these data to a de novo sequencing algorithm [19] resulted in the correct primary structure. Conventional and activated ion (AI) [9d] ECD and CAD spectra of cytochrome *c* showed cleavages between all but 9 of its 103 amino acid pairs, most of these in K_{13}–V_{20} region that contains the Fe^{3+} heme cyclic (14–17) side chain whose H· affinity is ~20 kcal/mol higher than that of the amide carbonyl [9b].

The S/N problem is epitomized in the AI ECD spectrum of the 43 kDa thiaminase, a mixture of 379, 380, and 381 residue proteins [9d]. To obtain many fragment ions of appreciable S/N requires electron irradiation until essentially all molecular ions are removed; as the number of products increases, their mass values decrease. Although the largest ECD products are $\boldsymbol{c}_{49}$ and $\boldsymbol{z}\cdot_{79}$, the ECD spectra define 55 cleavages in these terminal regions, including the sequence A^{14} to W^{33}, except for the $D–P^{22}$ doublet. Although the lack of products representing the central 69% of the protein could be due to insufficient ion activation to break its noncovalent bonding, the larger, more highly charged products must also be undergoing secondary electron capture (whose cross section is dependent on the square of the charge), with the process repeated to accumulate small terminal ***c*** and ***z***· products of sufficient S/N. Secondary e^- capture of a ***c*** (or ***z***·) ion forms another ***c*** (or ***z***·) ion plus an internal (***i***) ion. Such ***i*** ions are not found even in ECD spectra of peptides [17b]; although the AI ECD spectra of large proteins use far higher e^- currents, the probability of forming a specific ***i*** ion is far lower (formed by two cleavages, each with a probability of ~1/380). This plethora of ***i*** products could account for the continuum of near-background peaks of unit *m/z* spacing over *m/z* 900–1500 in some AI ECD spectra [9d]. Thus higher field FTICR instruments that can store and measure more ions should provide even more ECD sequence information for large proteins.

A serious problem in using MS/MS data from conventional energetic dissociation methods is assigning mass values as ***b***, ***y***, or ***i*** ions. ECD spectra do not contain appreciable *i* ions (vida supra), and the less abundant ***a***·, ***y*** products (eq 4, and often the ***b***, ***y*** eq 1 products) usually involve cleavages between the same amino acids as those yielding ***c***, ***z***· products. These coincidences ("golden complementary sets") do provide sequence ordering information. For products formed by cleavage between the same amino acids, $\boldsymbol{z}\cdot - \boldsymbol{y} = -16.02$ Da, $\boldsymbol{c} - \boldsymbol{b} = +17.03$ Da, and $\boldsymbol{c} - \boldsymbol{a}\cdot = +44.02$ Da (this can also be CO_2 loss), as set forth in detail in the description of the sequencing algorithm [19]. For example, if a complementary ***c***, ***z***· ion pair (products whose masses sum to that of the molecule) is accompanied by an ion of whose mass is 17.03 Da more than that of one of the pair, that one is a ***z***· ion and the accompanying ion is a ***y***. ECD, especially AI ECD, spectra often contain ***b***, ***y*** pairs from adventitious CAD; such pairs are similarly valuable for ordering ***c***, ***z***· complementary pairs [19]. For >20 kDa proteins, no complementary ECD fragment mass pairs are observed; even without these, the −16.02 and +17.03 Da mass differences can provide terminal assignments of good confidence. Also, any assignment as N- or C-terminal to a partial sequence automatically provides the opposite assignment to a second partial sequence containing overlapping mass values.

Sequence Tags

The proteomics explosion has greatly increased the need for the identification of proteins expressed from genes of known DNA sequence. MS is proving invaluable for this, but generally requires purification (2-D gels) to relatively high purity, digestion to small peptides, with mass spectrometry molecular weight determination. If these values do not retrieve a confident protein assignment from the database, MS/MS of one or more peptide ions is used to generate "sequence tags" [27a] for database retrieval. ECD offers a powerful alternative that should require less rigorous initial protein separation (e.g., capillary electrophoresis, capillary liquid chromatography), no proteolysis, with only CAD [27b] or ECD of the mass spectrometry-separated molecular ions from ESI of a mixture containing several proteins. AI ECD [9d] of carbonic anhydrase (29 kDa, 259 residues) gave nine sequence tags of six or more residues, with the largest of 16 residues. The largest for thiaminase contained 10 N-terminal residues, despite the S/N reduction due to formation of these ***c*** ions from 379, 380, and 381 amino acid proteins.

Conclusions

To date, ECD of large molecules has been implemented only with FTICR instrumentation [9c]; this is conceivable with other common mass spectrometry instruments, but overall ECD performance superior to FT-ICR appears doubtful. It is especially notable that ECD has greatly expanded the unimolecular chemistry of large multiply charged positive ions. Now odd-electron ions can be formed, and the extensive knowledge of small $OE^{+\cdot}$ ion chemistry [15] should be extendable to provide both new ionic reactions and new characterization capabilities for bio- and other macromolecules; copolymers appear to be a particularly promising application [20, 21]. For basic physical chemistry of ions, further investigation of the unique ECD mechanisms that appear to involve initial formation of a high Rydberg state and nonergodic dissociation, could greatly expand knowledge in this important research frontier. Finally, for the sudden critical importance of proteomics, ECD appears to provide a powerful new method for protein identification, correction of DNA sequence errors, characterization of post-translational modifications, and even de novo sequencing.

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